

A New Class of Isothiocyanate-Based Irreversible Inhibitors of Macrophage Migration Inhibitory Factor[†]

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ABSTRACT: Macrophage migration inhibitory factor (MIF) is a homotrimeric multifunctional proinflammatory cytokine that has been implicated in the pathogenesis of several inflammatory and autoimmune diseases. Current therapeutic strategies for targeting MIF focus on developing inhibitors of its tautomerase activity or modulating its biological activities using anti-MIF neutralizing antibodies. Herein we report a new class of isothiocyanate (ITC)-based irreversible inhibitors of MIF. Modification by benzyl isothiocyanate (BITC) and related analogues occurred at the N-terminal catalytic proline residue without any effect on the oligomerization state of MIF. Different alkyl and arylalkyl ITCs modified MIF with nearly the same efficiency as BITC. To elucidate the mechanism of action, we performed detailed biochemical, biophysical, and structural studies to determine the effect of BITC and its analogues on the conformational state, quaternary structure, catalytic activity, receptor binding, and biological activity of MIF. Light scattering, analytical ultracentrifugation, and NMR studies on unmodified and ITC-modified MIF demonstrated that modification of Pro1 alters the tertiary, but not the secondary or quaternary, structure of the trimer without affecting its thermodynamic stability. BITC induced drastic effects on the tertiary structure of MIF, in particular residues that cluster around Pro1 and constitute the tautomerase active site. These changes in tertiary structure and the loss of catalytic activity translated into a reduction in MIF receptor binding activity, MIF-mediated glucocorticoid overriding, and MIF-induced Akt phosphorylation. Together, these findings highlight the role of tertiary structure in modulating the biochemical and biological activities of MIF and present new opportunities for modulating MIF biological activities *in vivo*.

Macrophage migration inhibitory factor (MIF)¹ is a multifunctional protein that is now recognized as a major player in innate immune responses and regulation of the host response to inflammation and infection. MIF is expressed in a variety of cells and has been implicated in a wide range of cellular activities, including transcriptional regulation of inflammatory gene products (1), regulation of glucocorticoid activity (2), cell cycle control via promotion of cell proliferation and survival through activation of the ERK1/2 MAPKs (3), inhibition of the p53 and retinoblastoma/E2F tumor suppressor pathways (4, 5), and activation of the phosphoinositide 3-kinase (PI3K)/Akt survival pathway (6). Several lines of evidence support a central role for

MIF in the pathogenesis of inflammatory and autoimmune diseases, including arthritis (7, 8), glomerulonephritis (9, 10), diabetes (11, 12), colitis (13), sepsis (14–18), acute respiratory distress syndrome (19, 20), and cancer (21–23). Therefore, targeting MIF activity has emerged as a viable therapeutic approach for treating these inflammatory and autoimmune diseases.

In addition to its reported biological activities, MIF is known to catalyze the tautomerization of phenyl pyruvate (or hydroxyphenyl pyruvate) and D-dopachrome methyl ester (Figure 1) (24–27). Although the physiological substrates of MIF remain unknown, and the exact relationship between its catalytic activity and biological function is still not well understood, targeting MIF tautomerase activity using small-molecule inhibitors has emerged as a viable strategy for inhibiting MIF proinflammatory activity and attenuating its biological activity *in vitro* and *in vivo* (28–30). The first example of a small-molecule MIF inhibitor was NAPQI, which was described by Senter et al. to form a covalent complex with the MIF catalytic proline residue (Pro1) (Figure 1), thereby eliminating tautomerase activity. NAPQI-modified MIF failed to override the immunosuppressive effect of dexamethasone on LPS-induced TNF production by monocytes but has not been tested in the clinic because of its potential toxicity (31). Al-Abed and co-workers subsequently developed several active site inhibitors based on

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¹Abbreviations: MIF, macrophage migration inhibitory factor; ITC, isothiocyanate; BITC, benzyl isothiocyanate; AITC, allyl isothiocyanate; EITC, ethyl isothiocyanate; MITC, methylallyl isothiocyanate; 2PITC, piperidinoethyl isothiocyanate; CPITC, cyclopropyl isothiocyanate; PEITC, phenylethyl isothiocyanate; CD, circular dichroism; SEC, size-exclusion chromatography; AUC, analytical ultracentrifugation; MALDI, matrix-assisted laser desorption ionization; HSQC, heteronuclear single-quantum coherence; Wt, wild type; PDB, Protein Data Bank.

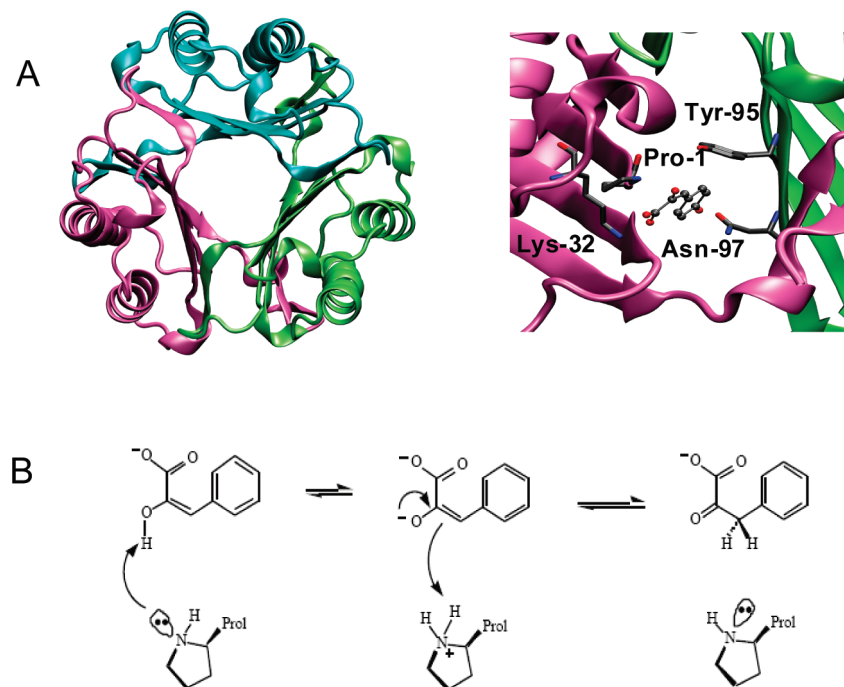


FIGURE 1: Trimer formation is required for MIF tautomerase activity. (A) Ribbon diagram showing the MIF homotrimer and the tautomerase active site. Each monomer is indicated by a different color. The figure was generated using VMD, and PDB entry 1GDO has a resolution of 1.5 Å. The MIF catalytic site complexed with (*E*)-2-fluoro-*p*-hydroxycinnamic acid indicates that the molecules interact through the side chains of Pro1, Lys32, and Asn97 from two neighboring subunits. (B) Mechanism of MIF tautomerase reaction as proposed by Stamps et al. (27).

modifications of the scaffold of (*S,R*)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) (32, 33). MIF tautomerase inhibitors, including ISO-1, phenolic hydrazone (34), OXIM-11 (29), and COR10014 (30), were shown to have protective effects in animal models of sepsis and RA. Inhibition of MIF tautomerase activity by these molecules was also accompanied by modulation of its biological activities, including inhibition of (1) MIF glucocorticoid overriding activity and (2) endotoxin (LPS)-induced TNF production and MIF-mediated (i) stimulation of ERK1/2 MAP kinase and proliferation of serum-starved cells (33), (ii) upregulation of arachidonic acid in macrophages, and (iii) Cox-2 activation.

Herein we report a new class of ITC-based irreversible inhibitors of MIF. To elucidate their mechanism of action, we performed detailed biochemical, biophysical, and structural studies to determine their effect on the conformational state, quaternary structure, enzymatic activity, receptor binding, and biological properties of MIF. These studies demonstrate that in addition to blocking the catalytic activity of MIF, selective modification of Pro1 alters the tertiary structure of MIF and results in a significant reduction in the level of MIF-mediated glucocorticoid overriding and MIF-induced Akt phosphorylation. These findings and their implication for therapeutic strategies targeting MIF are presented and discussed.

MATERIALS AND METHODS

Chemicals. Benzyl isothiocyanate (BITC), allyl isothiocyanate (AITC), ethyl isothiocyanate (EITC), methallyl isothiocyanate (MITC), 2-piperidinoethyl isothiocyanate (2PITC), cyclopropyl isothiocyanate (CPITC), and phenylethyl isothiocyanate (PEITC) were purchased from Sigma or Fluka and were of the highest purity available.

Expression and Purification of Human MIF. MIF protein was expressed by heat shock transformation of the BL21DE3

Escherichia coli strain (Stratagene) with bacterial expression vector pET11b containing the human MIF gene under control of the T7 promoter. Four hours postinduction, the cells were harvested, resuspended in lysis buffer (50 mM Tris, 50 mM KCl, 5 mM MgAc, and 0.1% azide), sonicated at a 200 Hz pulse repetition frequency for 20 min using a VibraCell™ sonicator, and harvested by centrifugation at 13000g for 25 min. The clarified cell lysate was filtered, injected onto a MonoQ anion exchange column (HiPrep 16/10 Q FF, GE Healthcare), and eluted with a linear NaCl gradient in elution buffer [25 mM Tris-HCl (pH 7.4) and 150 mM NaCl]. The flow-through fractions containing MIF were pooled and loaded onto a Superdex 75 16/60 (HiLoad 16/60, Superdex 75, Pharmacia Biotech) gel filtration column. Fractions corresponding to MIF were pooled together, dialyzed against PBS, and filtered. The identity and purity of the protein were confirmed by MALDI-TOF mass spectrometry, silver staining, and Western blotting using the rabbit anti-MIF antibody from Zymed (Invitrogen) at a dilution of 1:20000. When required, the proteins were concentrated using a concentrator with a molecular mass cutoff of 5 kDa and stored at 4 °C until they were used. Uniformly ¹⁵N-labeled protein samples were prepared for NMR experiments by culturing the bacteria in M9 minimal medium containing [¹⁵N]ammonium chloride (1 g/L) as the only nitrogen source. Recombinant MIF used for cellular studies was subjected to LPS removal as described previously (35). Briefly, Bacterial cell lysate was injected into an anion exchange column; the flow-through fractions containing MIF were applied to C8-Septak cartridge, and MIF was eluted with a 60% acetonitrile/water mixture, lyophilized, and renatured. The LPS content was quantified using a chromogenic *Limulus amoebocyte* assay.

Analytical Ultracentrifugation (AUC). Analytical ultracentrifugation experiments were performed using purified and dialyzed MIF samples (10–20 μM) preincubated with BITC for 1 h at an MIF concentration corresponding to 100% inhibition.

Radial UV scans were recorded on a Beckman Optima XL-A instrument at a wavelength of 277 nm. Sedimentation velocity experiments were conducted at 20 °C using 380–400 μL of protein solution. Data were recorded at a rotor speed of 50000 rpm in continuous mode, with a step size of 0.003 cm. The experimentally determined partial specific volume of 0.765 mg/mL was used to calculate the molecular masses of MIF (36). The sedimentation velocity profiles were analyzed as a $c(s)$ distribution of the Lamm equation using SEDFIT. Molar mass distributions $c(M)$ were obtained by transforming the corresponding $c(s)$ using SEDFIT.

Analytical Size Exclusion Chromatography and Light Scattering. Size exclusion chromatography experiments were performed on purified MIF incubated in the presence and absence of BITC or its analogues using an analytical Superdex 75 10/30 column at room temperature. The column was equilibrated with 25 mM Tris buffer (pH 7.6), and MIF samples (12 μM in 1 \times PBS) in volumes of 0.5 mL were injected onto the column and eluted at a flow rate of 0.4 mL/min in an Agilent HPLC system attached to both a UV, refractive index and a DAWN HELLIOS multiangle light scattering detector (Wyatt Technology Corp., Santa Barbara, CA). Absolute molecular masses were determined using ASTRA version 5.3 from Wyatt Technology.

MALDI-TOF-MS Molecular Mass Measurements for Examining Possible Protein Modifications. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed to examine any HITS-induced protein modification. We prepared the matrix solution by dissolving a 14 mg/mL sinapinic acid (SA) solution in a 0.1% TFA/ACN mixture (1:1). A thin matrix layer was generated on the mirror-polished target using a gel loading tip. One microliter of sample (15 μM MIF incubated with 10 μM HITS for 1 h) was mixed with 1 μL of SA matrix solution, and 0.8 μL of this mixture was deposited on top of the thin layer and allowed to air-dry. The samples were analyzed with a 4700 MALDI-TOF/TOF instrument (Applied Biosystems).

Protein Digestion Analysis for Identifying the Modified Residue. One microliter of the MIF sample previously incubated for 1 h with 10 μM BITC was diluted in 5 μL of 100 mM BA and digested overnight with trypsin (Promega). The digestion was stopped with 1 μL of 10% TFA and the mixture stored at 4 °C until further use. The samples were analyzed by MALDI-TOF-MS on a 4700 MALDI-TOF/TOF instrument (Applied Biosystems) or an Axima CFR plus instrument (Shimadzu) without further purification. One microliter of sample was mixed with 1 μL of DHB [20 mg/mL in a 1% PA/ACN mixture (1/1)], and 0.8 μL of this mixture was deposited on the target and allowed to air-dry. MALDI matrices and calibrants were obtained from Sigma/Fluka (Schneidorf, Germany). TFA was from Pierce. SA and 2,5-dihydroxybenzoic acid (DHB) were from Fluka. Cytochrome *c* and apomyoglobin were from Sigma.

Nuclear Magnetic Resonance (NMR) Spectroscopy. NMR spectra were recorded at 27 °C on a Bruker Avance 600 MHz NMR spectrometer using a triple-resonance cryoprobe equipped with *z*-axis self-shielded gradient coils. Measurements were performed using 100–200 μM samples. All samples were prepared in 20 mM Na_2HPO_4 , 0.5 mM EDTA, 0.02% NaN_3 (pH 7.0), and 10% D_2O . Two-dimensional ^1H – ^{15}N heteronuclear single-quantum coherence (HSQC) spectra were recorded using 256×1024 complex data points in the F_1 and F_2 dimensions, with a relaxation delay of 1.0 s. Sixty-four scans per increment were recorded for each spectrum. Spectral widths were 2200 and

8950 Hz in the ^{15}N and ^1H dimensions, respectively. Specifically, we recorded two-dimensional ^1H – ^{15}N HSQC spectra to follow chemical shift changes upon addition of inhibitory compounds and to map the interaction surface of MIF for each compound. For this, a reference spectrum of MIF alone was recorded in the presence of 1% DMSO. For each titration step of MIF with inhibitory compounds, a new sample was prepared in which MIF was mixed with the corresponding compound dissolved in DMSO, with the DMSO concentration maintained at 1%.

Spectra were processed with Topspin (Bruker) and NMR-Pipe (37). Visualization and manipulation were performed using the public domain graphics program Sparky 3 (59). Resonance assignments were previously published for the same buffer system at 30 °C (38). The addition of 1% DMSO and a small change in temperature did not interfere with the transfer of resonance assignments. Both intensity and chemical shift differences were analyzed with respect to the reference spectrum. Mean weighted ^1H – ^{15}N chemical shift differences were calculated according to the relationship $\Delta\delta = \{[(\Delta\delta^1\text{H})^2 + [(\Delta\delta^{15}\text{N})/5]^2]^{1/2}\}/2$ (39). Changes were mapped on the crystal structure using PDB entry 1GDO (1.5 Å resolution) and PyMOL.

MIF Receptor Binding Assay. Recombinant, soluble huMIF receptor ectodomain (sCD74^{73–232}) was cloned and purified as described previously (40) and used to coat 96-well plates by incubation at 4 °C overnight. Plates were washed four times with TTBS (pH 7.4) and blocked with superbloc buffer (Pierce) for 1 h at room temperature. Biotinylated huMIF (Roche Molecular Biochemical) was prepared and added together in triplicate wells with increasing concentrations of purified huMIF trimer (35), heat-denatured huMIF, or test MIF proteins. Incubation took place at room temperature for 2 h. After wells had been washed four times with TTBS (pH 7.4), streptavidin-conjugated alkaline phosphatase (R&D) was added and incubation continued for an additional 1 h, and the wells were washed and bound complexes detected via addition of *p*-nitrophenyl phosphate (Sigma). Absorbance was read at 405 nm using a DYNATECH, MR5000 plate reader, and values were plotted as percent OD₄₀₅ relative to wells containing biotinylated MIF alone. The plots shown are representative of at least three assays, and each plotted point depicts a standard error of the mean of $\leq 10\%$.

Glucocorticoid Overriding Assay. PBMCs from healthy donors (recruited by the Blood Center, Lausanne, Switzerland) were purified with a Ficoll-Hypaque density gradient (GE Healthcare). To obtain macrophages, adherent PBMCs (10^6 cells per well in 48-well plates) were cultured for 6 days in RPMI 1640 with Glutamax. Media were supplemented with 10% heat-inactivated FCS (Seromed) and antibiotics. MIF was incubated with the inhibitors for 15 min at room temperature. Primary macrophages were preincubated for 1 h with 100 nM dexamethasone, dexamethasone with MIF (100 ng/mL), or dexamethasone with MIF and BITC (10 μM) before the addition of 100 ng/mL *Salmonella Minnesota* Ultra Pure LPS (List Biologicals Laboratories). TNF concentrations in cell culture supernatants collected after 4 h were measured with a bioassay. Statistical analyses were performed using one-way ANOVA followed by Scheffé's test for BITC treatment from four independent experiments. A $p < 0.05$ significance was required for rejection of the null hypothesis.

Phospho Akt Activation by MIF. Confluent HeLa cells were incubated for 2 h with recombinant human MIF (50 ng/mL) in the presence of BITC, LY294002 (10 and 1 μM), or DMSO. Phosphorylation of Akt (Ser473) was assessed using an Alpha

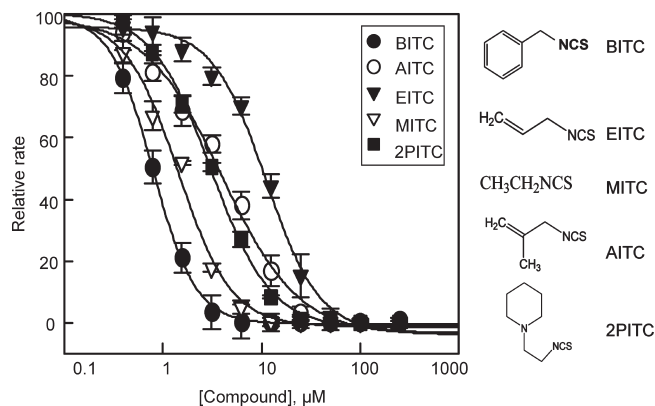


FIGURE 2: Several isothiocyanate-based compounds inhibit MIF tautomerase activity in a concentration-dependent manner. IC_{50} concentration response curve (mean \pm standard deviation; $n = 8$) obtained by monitoring the initial velocity of tautomerization of the HPP keto form to the HPP enol form by MIF. MIF (100 nM) was preincubated with different concentrations of ITC analogues BITC, AITC, EITC, MITC, and 2PITC for 5 min, followed by the addition of 2 mM hydroxyphenyl pyruvate, and then the absorbance was monitored at 300 nm for 5 min. The initial velocity was calculated and normalized to the percentage of activity.

screen SureFire phosphokinase kit, an immunosandwich assay based on alpha screen technology in two incubation steps, according to the manufacturer's protocol. First, cell lysate was incubated for 2 h with an anti-Akt antibody captured on protein A-conjugated acceptor beads. Then, the biotinylated anti-pAkt antibody was added with streptavidin-coated Alpha donor beads. The two beads were brought into the proximity of each other only in the presence of phosphorylated Akt. Upon illumination at 680 nm, the Alpha donor bead photosensitizer converts the ambient oxygen to an excited form, which will enable the excitation of the Alpha acceptor beads. Data are reported as Alpha screen counts. Statistical analyses were performed using one-way ANOVA followed by Scheffé's test for compound treatment from three independent experiments. A $p < 0.05$ significance was required for rejection of the null hypothesis.

RESULTS

Benzyl Isothiocyanate (BITC) Inhibits MIF Tautomerase Activity. During our screening for inhibitors of MIF tautomerase activity, we observed that BITC demonstrated 100% inhibition of the tautomerase activity of both human and murine MIF at concentrations of 10 μ M. We hypothesized that this inhibitory activity is due to the reactivity of the isothiocyanate group in BITC. To test this hypothesis, we evaluated the inhibitory effect of several ITCs at concentrations ranging from 0 to 100 μ M and obtained IC_{50} values 0.79, 3.69, 11.10, 1.43, and 3.09 μ M for BITC, AITC, EITC, MITC, and 2PITC, respectively (Figure 2).

BITC Mode of Action. Inhibition of MIF's tautomerase activity can occur via at least five different mechanisms: (1) binding to the active site, (2) allosteric inhibition, (3) covalent modification of active site residues, (4) disruption of the active site through compound-induced dissociation of the active trimer, and (5) stabilization of the MIF monomer and prevention of its reassociation to form the active trimer. To elucidate the mechanism of action of BITC, we performed detailed biochemical, biophysical, and structural studies to determine the effect of BITC and its analogues on the conformational state, quaternary

structure, catalytic activity, receptor binding, and biological activity of MIF.

BITC Inhibition of MIF Tautomerase Activity Is Mediated by Selective Modification of the N-Terminal Proline. BITC contains the isothiocyanate reactive group which can readily interact with O-, S-, or N-based nucleophiles. As a result, BITC may react with cysteine residues (41, 42) or with the N-terminus of the protein, as in the case of phenyl isothiocyanate (PITC, Ph-N=C=S) used in protein sequencing using Edman degradation. This raised the possibility that BITC inhibition of MIF tautomerase activity might occur through covalent modification of MIF. To test this, MIF (15 μ M) in 50 mM Tris buffer (pH 7.4) was incubated with BITC (10 μ M) for 60 min and the formation of a BITC-MIF adduct was monitored by MALDI-TOF-MS. Under these conditions, a shift in the monomeric molecular mass of MIF by 148.33 Da from 12345.16 to 12493.50 Da was observed, which corresponds to the addition of one molecule of BITC. At concentrations of $> 10 \mu$ M, we observed complete conversion to the singly modified form of MIF, consistent with a single-site modification by BITC (Figure 3A and Figure S1 of the Supporting Information).

To identify the site of covalent modification, we subjected the BITC-modified MIF to proteolytic digestion by trypsin and peptide mapping by mass spectrometry. Tryptic digestion of BITC-modified MIF (Figure 3A) revealed a fragment peptide with an m/z ratio of 1436.70, which corresponds to the N-terminal fragment (PMFIVTNTNVPR, m/z 1287.61) with one molecule of BITC (Figure 3B). This fragment is devoid of any nucleophilic residue (lysines and cysteines) that may react with ITCs, except for the N-terminal proline. MS/MS analysis of this peptide fragment demonstrated that BITC modification takes place at the N-terminal proline residue. Six additional ITC analogues, AITC, EITC, MITC, 2PITC, CPITC, and PEITC, were also tested, and all were shown to modify MIF at the N-terminal Pro residue (Figure 3C).

BITC Modification Is Specific to MIF. BITC shares structural similarities with PITC, which is used in N-terminal polypeptide sequencing, suggesting that its modification of MIF might not be specific. To assess this possibility, different proteins were incubated with BITC and characterized by MALDI-TOF. BITC was incubated with ubiquitin, lysozyme, or α -synuclein at equimolar concentrations either for 1 h or overnight at room temperature. No modification was detected for either incubation time by MS, suggesting that the reactivity with N-terminal proline is specific and likely due to its altered pK_a and increased nucleophilicity (Figure S2 of the Supporting Information).

BITC Modifies Only Catalytically Active Forms of MIF. Recent studies from our laboratory demonstrated that disrupting the conformation of the C-terminus via truncation or insertion of secondary structure-disrupting elements (proline residues) within the C-terminal region (residues 104–115) abolished the protein's activity without altering its oligomerization state. With these constructs at our disposal, we were interested in further probing the specificity of BITC-induced modification of MIF by determining whether BITC selectively modifies enzymatically active MIF. Six trimeric MIF variants [three enzymatically active (Wt, R73A, and N110C) and three inactive (R73A Δ 5, R73A Δ 10, and P107)] were tested via incubation with BITC. As expected, MALDI-TOF analysis revealed total modification of the enzymatically active forms of MIF (Wt, N110C, and R73A), whereas no modification was observed

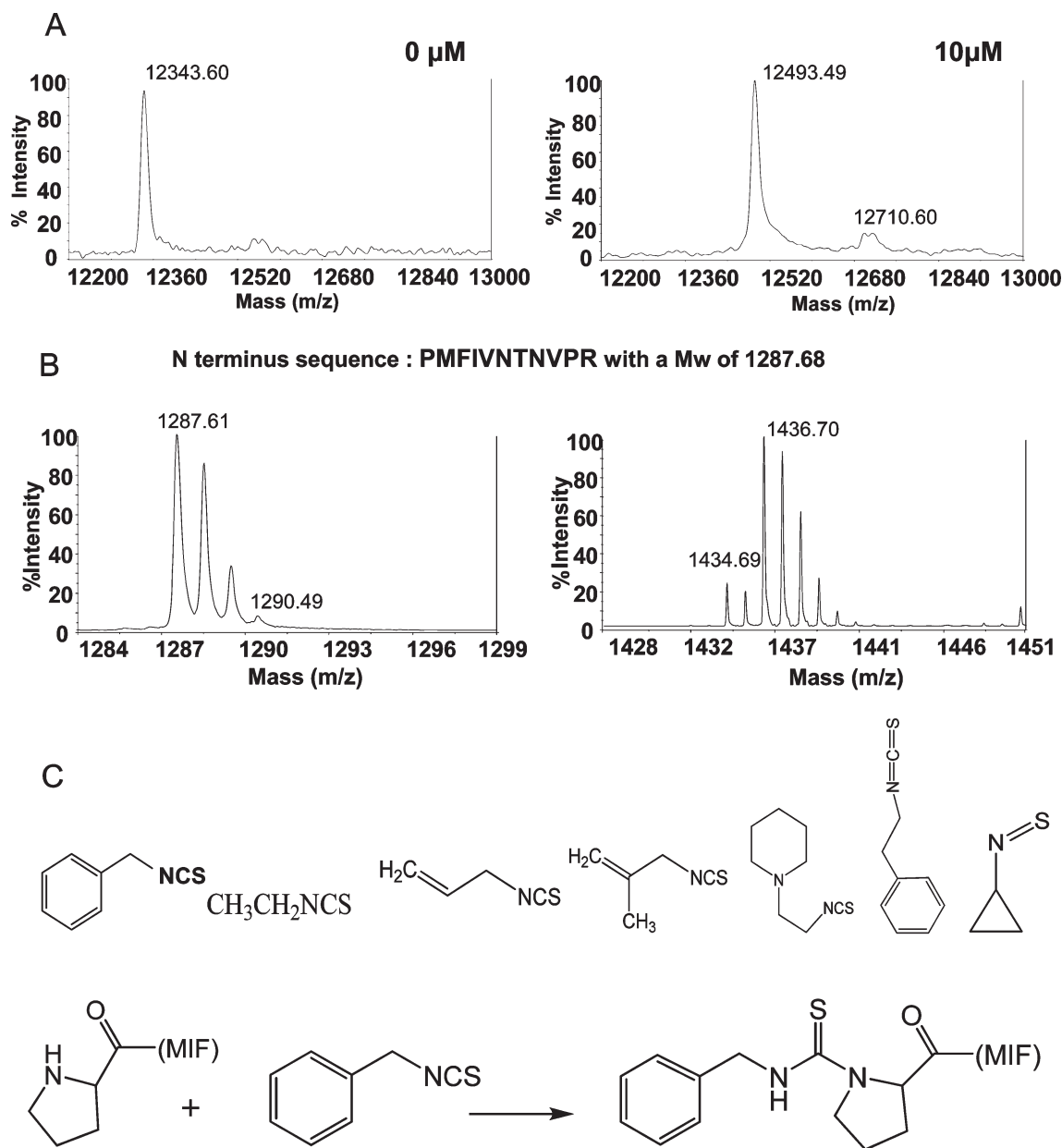


FIGURE 3: BITC and other isothiocyanate-based MIF inhibitors covalently modify the catalytic N-terminal proline residue (Pro1). (A) MIF was incubated in the presence or absence of BITC or one of its analogues (10 μM) for 30 min, and the samples were then prepared using sinapinic acid as a matrix and analyzed by MALDI-TOF/TOF in linear positive ion mode. (B) MIF was incubated with 10 μM BITC for 60 min and digested with trypsin 1/50 ON at 37 $^\circ\text{C}$ and pH 8. After being desalted, the samples were analyzed by MS. (C) Schematic depiction of the chemical reaction leading to the formation of a covalent adducts between Pro1 of MIF and BITC.

for inactive MIF proteins (R73A Δ 5, R73A Δ 10, and P107), suggesting that BITC modifies only catalytically active trimeric forms of MIF (Figure 4).

Modification by BITC and Other ITCs Does Not Alter the Oligomerization State of MIF. To determine whether any of the ITCs function by disrupting the MIF trimer, we compared the quaternary structure distribution of MIF in the presence or absence of BITC (6 μM) by sedimentation velocity analytical ultracentrifugation, size exclusion chromatography (SEC), and light scattering (Figures 5 and 6). MIF preincubated either with DMSO as a control or with BITC sedimented predominantly as a single species with a sedimentation coefficient and molecular mass corresponding to those of the trimer (Figure 5). Previous studies from our laboratory demonstrated that MIF elutes as two peaks in the SEC that correspond to two different conformations of the trimers (43). The two different conformational states of the

trimer differ mainly in their tertiary structure. To determine if Pro1 modification by the ITCs affects the distribution of the different trimers, we compared the elution profiles and molecular mass distribution of MIF before and after modification by ITCs by online SEC coupled to a multiangle light scattering detector. Figure 6 demonstrates that modification of Pro1 by all active ITC MIF inhibitors resulted in a shift in the elution profile of MIF. In each case, the covalently ITC-modified MIF elutes as a single peak at 28 min, whereas the unmodified MIF elutes as a mixture of two overlapping peaks (31 and 33 min). The light scattering results demonstrate that this shift in the SEC profile does not correspond to a change in oligomerization state and is likely to be due to differences in tertiary structure between the modified and the unmodified trimer forms of MIF. In each case, the ITC-modified MIF exhibited a molecular mass corresponding to the trimer. These findings provide strong evidence in support of our

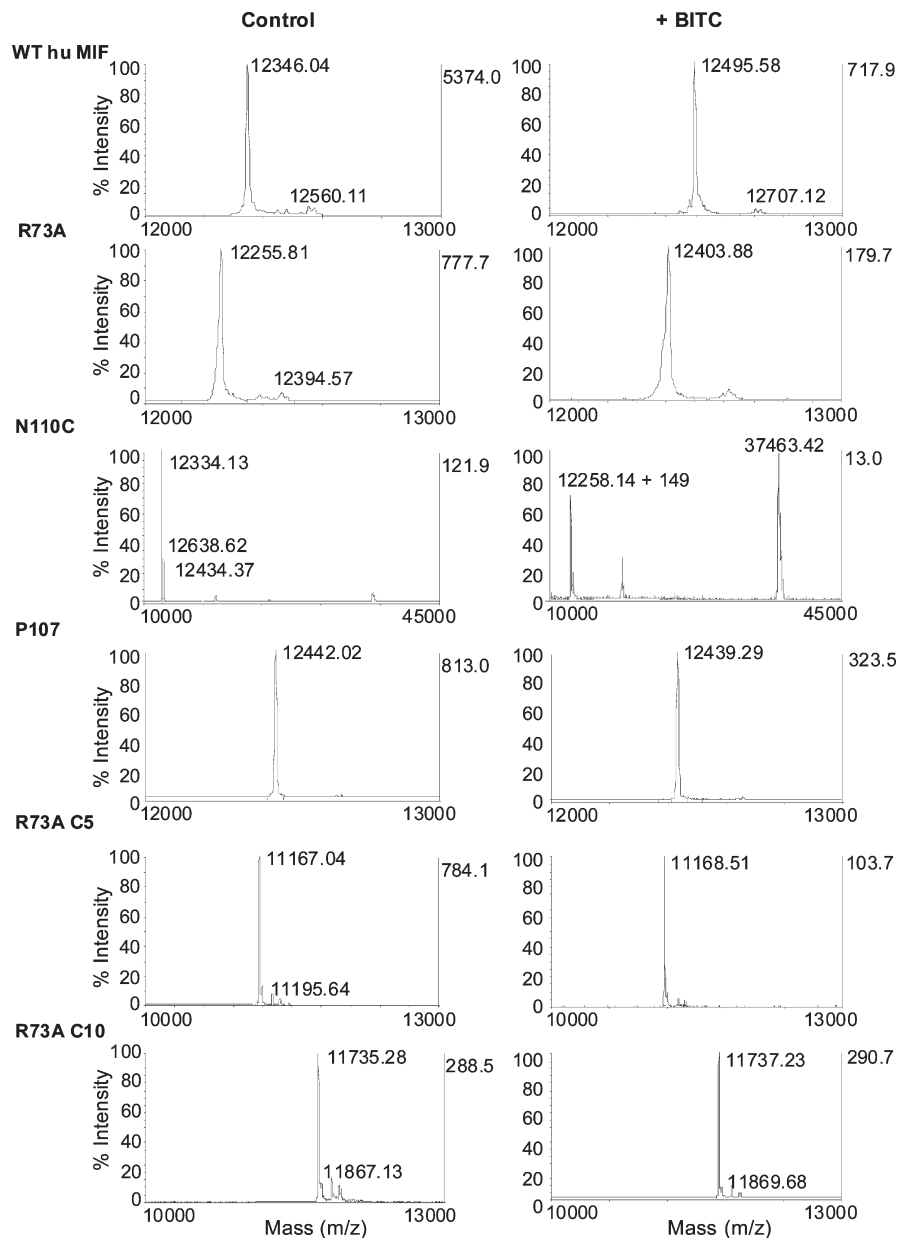


FIGURE 4: Only enzymatically active forms of the MIF trimer undergo covalent modification by BITC and its active analogues. Six trimeric MIF variants [three enzymatically active (Wt, R73A, and N110C) and three inactive (R73A Δ 5, R73A Δ 10, and P107)] were tested via incubation with 10 μ M BITC for 60 min, and the samples were then analyzed by MALDI-TOF/TOF.

hypothesis that the MIF trimer exists in different conformational states.

Elucidating the Structural Basis for the Inhibition of MIF Enzymatic Activity Using NMR. To further elucidate the structural basis for the inhibition of MIF enzymatic activity, NMR titrations were performed with BITC. An equimolar mixture of MIF with BITC results in severe resonance broadening throughout the whole sequence, indicative of conformational exchange. However, more drastic effects are seen for residues 3, 4, 32, 35–39, 49, 50, 58–67, 79, 84–89, 95–97, 103, 107–109, and 112–114 (Figure 7A,B). The majority of these residues cluster around P1 and are components of the active site (Figure 7C). Similar effects had been previously observed in an NMR titration of MIF with HPP (44). Additionally, Y95 and N97 are components of the active site. The subunit interface is not as hydrophobic as the interior of the monomer; however, there is a hydrophobic patch on the surface. Residues Y36, Y95, W108, and F113 are part of this patch.

Inhibitors of MIF Tautomerase Activity Interfere with Binding of MIF to Its Receptor, CD74. A major cellular action of MIF is to inhibit activation-induced apoptosis by inducing sustained MAP kinase (ERK1/2) activation (3, 23). MIF-dependent ERK1/2 phosphorylation proceeds by engagement of a two-component receptor complex that comprises the binding protein, CD74, and the signal initiating component CD44 (40, 45). Because the tautomerase site has been implicated in receptor binding (31, 46), we tested the ability of selected tautomerase inhibitors to inhibit the interaction of MIF with the soluble CD74 ectodomain (CD74^{73–232} or sCD74) using an ELISA-based MIF “capture” assay (47). We tested the potential inhibitory action of the N-terminal catalytic site modifier, BITC, as well as the isothiocyanate-based congeners, AITC, EITC, MITC, and 2PITC (Figure 8). With the possible exception of 2PITC, these compounds exhibited no significant dose-dependent effect at the concentrations tested, and inhibition plateaued at approximately 30–35%. Taken together, these data suggest

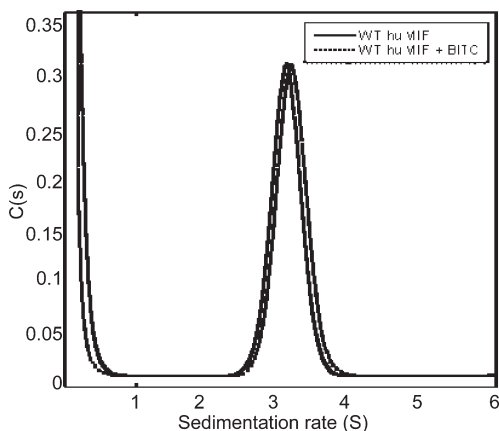


FIGURE 5: Covalent modification of MIF by BITC does not affect its oligomerization state. Sedimentation velocity profiles of MIF in the presence or absence of BITC. MIF (10 μ M) in PBS was preincubated with 6 μ M BITC for 1 h at room temperature. Sedimentation coefficient distributions were obtained by analysis of the sedimentation profiles using the $C(s)$ distribution as a variant of Lamm equation solutions. Calculations were performed using SEDFIT (www.sedfit.com).

that that these compounds impart sufficient structural alterations on MIF to influence its interaction with CD74.

BITC Inhibits MIF-Mediated Glucocorticoid Overriding Activity and Enhancement of Akt Phosphorylation. Among the important *in vivo* immunoregulatory functions of MIF is its ability to counter-regulate the immunosuppressive effect of glucocorticoids on proinflammatory cytokine production (2). To examine whether BITC interferes with the immunoregulatory function of MIF, human macrophages were pretreated with dexamethasone (100 nM) with or without recombinant MIF (100 ng/mL) and BITC (10 μ M) prior to stimulation with LPS. Whereas dexamethasone inhibited TNF production by LPS-stimulated monocytes, preincubation with MIF overcame dexamethasone inhibition of TNF production (Figure 9A). Interestingly, incubation of MIF with BITC before addition to dexamethasone abrogated MIF-mediated dexamethasone overriding. It is noteworthy that BITC decreased TNF production even more potently than dexamethasone, suggesting that BITC has anti-inflammatory activity that is independent of its effect on MIF structure and tautomerase activity (Figure 9A). Similar results were obtained using the RAW 264.7 mouse macrophage cell line. To rule out the possibility that the observed effects were due to BITC toxicity, we verified that BITC did not alter macrophage viability under the culture conditions used to induce TNF production (data not shown).

Previous studies have shown that MIF promotes cell survival through activation of the PI3K/Akt pathway (48). The addition of recombinant MIF enhanced Akt phosphorylation via PI3K in NIH3T3 and HeLa cells (48). To determine whether BITC antagonized MIF-mediated Akt phosphorylation, we quantified the level of phosphorylated Akt in HeLa cells cultured for 2 h with MIF (50 ng/mL) preincubated with BITC or not. As shown in Figure 9B, MIF increased 1.5-fold the level of phosphorylated Akt in HeLa cells. BITC (1 and 10 μ M) inhibited MIF-induced Akt phosphorylation. The effect of BITC was specific for MIF, as BITC alone did not modify baseline Akt phosphorylation in HeLa cells. PI3K selective inhibitor LY294002, a known potent inhibitor of Akt, was used as a control. Overall, these data indicated that BITC was a powerful inhibitor of MIF biological activity.

DISCUSSION

Irreversible inhibition of MIF enzymatic activity via covalent modification of the N-terminal catalytic proline (Pro1) has been described for several MIF inhibitors, including 2-oxo-4-phenyl-3-butynoate (2-OB) (49), acetaminophen metabolites and related analogues (31), and 4-iodo-6-phenylpyrimidine (4IPP) (50). 2-OB also was shown to modify the catalytic N-terminal proline of 4-oxalocrotonate tautomerase (4-OT), a member of the tautomerase superfamily that shares structural and functional similarities with MIF. Herein we also show that irreversible modification of MIF by BITC abolished MIF tautomerase activity, reduced the level of binding of MIF to the CD74 receptor, and resulted in a significant reduction in the level of MIF-induced Akt phosphorylation. Different alkyl and arylalkyl ITCs were shown to exhibit similar effects, suggesting that the inhibitory effect of ITCs is mediated by the reactive isothiocyanate group. Although ITCs are known to react with lysine and cysteine residues, MIF contains three cysteines and three lysine residues per monomer; using proteolytic digestion and peptide mapping, we did not observe any modifications at these residues and could identify modifications only at Pro1. These results can be explained by the fact that the pK_a of Pro1 is reduced by 5 pH units (27), making it the most nucleophilic residue at physiological pH.

Isothiocyanates (ITCs) are abundant in cruciferous vegetables (e.g., broccoli, kale, brussel sprouts, cabbage, mustard, garden cress, cauliflower, and water cress). Epidemiological studies have shown that consumption of high levels of ITCs may protect against lung, colon, and other types of inflammation-related carcinogenesis, including skin cancer (51). ITCs, including BITC, have also been shown to inhibit cell proliferation, induce cell cycle arrest and apoptosis, and exhibit anticancer properties in different animal and cellular models of cancer (51–55).

To elucidate the molecular and structural basis underlying the inhibitory effect of BITC, we performed detailed biochemical, biophysical, and structural studies to determine the effect of Pro1 modification by BITC and other ITCs on the conformational state, quaternary structure, enzymatic activity, receptor binding, and biological properties of MIF. Together, our results demonstrate that covalent modification of Pro1 by BITC and other ITCs results in significant changes in the tertiary, but not the quaternary, structure of the MIF trimer. NMR studies show that BITC induces drastic effects on the tertiary structure of MIF, in particular, residues 3, 4, 32, 35–39, 49, 50, 58–67, 79, 84–89, 95–97, 103, 107–109, and 112–114 that cluster around Pro1 and constitute the tautomerase active site (Figure 7C), which explains its diminished tautomerase activity. BITC-modified MIF exists predominantly as a single species corresponding to the trimer (by SEC, AUC, and light scattering) and eluted via SEC as a single peak at 28 min, whereas the unmodified protein elutes as a mixture of two overlapping peaks at 31 and 33 min, both corresponding to the MIF trimer. These changes in tertiary structure translated into a reduction in MIF biological activity, i.e., binding to CD74 MIF receptor, MIF overriding of glucocorticoid-mediated inhibition of TNF production by macrophages, and MIF-induced Akt phosphorylation in HeLa cells.

Conformational Changes, Rather Than the Loss of Catalytic Activity, May Explain the Biological Consequences of Modifying the Catalytic Activity of MIF. Although the relationship between the enzymatic and biological activity of MIF remains unclear (32, 56), several small-molecule inhibitors

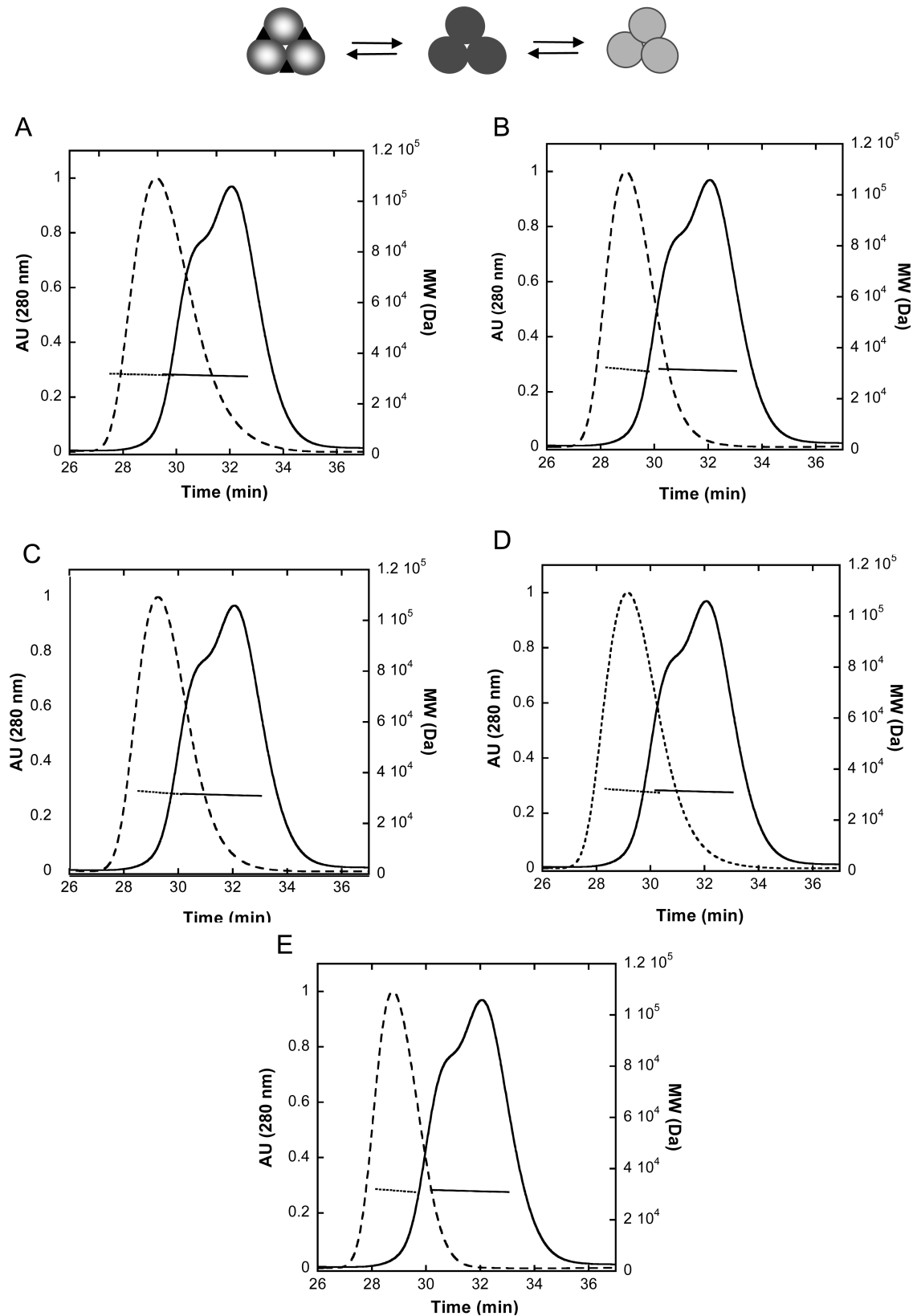


FIGURE 6: ITC modification of MIF stabilizes a trimeric form that is distinct from those adopted by the wild-type protein. MIF alone (—) and MIF incubated (---) with 10 μ M MITC (A), 2PITC (B), AITC (C), BITC (D), and EITC (E) were injected into an analytical Superdex 75 column and analyzed by light scattering upon elution. The individual data points show the molecular masses at each point in the chromatogram. Measurements were performed at a protein concentration of 10 μ M, in 1 \times PBS at pH 7.4.

of MIF tautomerase activity have been shown to protect against the proinflammatory effects of MIF *in vitro* and *in vivo* (28, 33, 34). These findings have been used to support the hypothesis that the tautomerase activity of MIF plays a direct role in modulating

its biological activities *in vivo*. However, several lines of evidence argue against this hypothesis and suggest that inhibition of MIF tautomerase activity is not sufficient to abolish its proinflammatory function. (1) Several catalytically inactive mutants of MIF

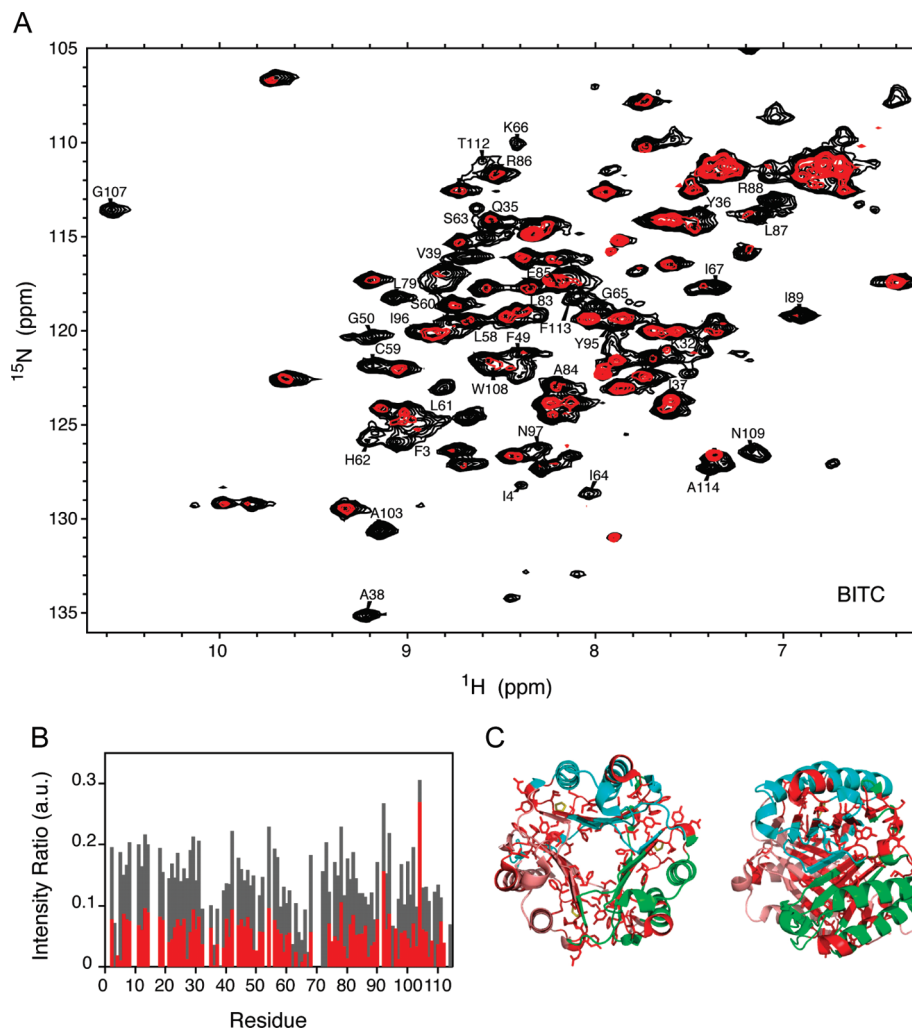


FIGURE 7: Covalent modification of Pro1 by BITC induces drastic changes in the tautomerase active site of MIF. (A) Superimposed contour plots of the ^1H - ^{15}N HSQC spectra of ^{15}N -labeled MIF in the free state (black) and for an equimolar mixture of MIF and BITC (red) measured at 27 °C. Addition of BITC to MIF results in severe resonance broadening. (B) Changes in individual cross-peak intensities of MIF (100 μM) upon addition of 50 (gray) and 100 μM (red) BITC. (C) Residues affected upon addition of BITC mapped into the crystal structure of the MIF homotrimer (PDB entry 1GDO). Residues that are affected cluster around P1 (red). The two views are related to each other by an angle of 90°.

retain their proinflammatory activity *in vitro*. (2) C-Terminal deletion of 5–10 C-terminal amino acids (residues 104–114) abolishes the catalytic activity of MIF without affecting its binding to the receptor CD74 (43) which plays a key role in modulating the signal transduction activity of MIF. (3) The catalytically inactive P1G mutant retains its binding to the MIF cell surface receptor (CD74) and intracellular binding protein JAB1/CSN5, albeit with some loss of affinity (56). (4) Studies on the P1G-MIF knockin mice (*mif*^{P1G/P1G}) demonstrate that the tautomerase activity of MIF is not essential for its growth regulatory properties (56).

These two hypotheses can be reconciled by the possibility that small-molecule interactions or covalent modification of the active site induces conformational or quaternary structural changes that modify protein–protein interactions that mediate MIF biochemical and biological properties. Given that MIF is trimeric, it is reasonable to consider that changes in the oligomerization state of the protein may underlie its multifunctional properties. To date, however, all the X-ray structure studies and *in solution* characterization of MIF using different techniques reveal that MIF exists as a stable trimer, and no evidence of a stable dimeric or monomeric MIF has been reported. It is noteworthy that the concentration of native MIF is in the range

of 4–16 nM. Therefore, we cannot rule out the possibility that there is a substantial amount of monomeric MIF under physiological conditions. Although our data suggest that the monomer is unstable and has a strong propensity to aggregate, it is possible that under physiological conditions the monomer is stabilized by its interactions with small molecules, peptides, or proteins in the cell.

We propose that the conformational heterogeneity, rather than quaternary structure, of MIF plays a role in modulating its protein–protein interaction and biochemical and biological activity and underlies its multifunctionality. This hypothesis is consistent with recent studies from our group demonstrating that the MIF trimer in solution exists in two different conformational states that differ primarily at the level of tertiary structure (F. El-Turk et al., unpublished data). These solution studies are supported by recent X-ray crystallography studies of the two *Leishmania major* orthologues of MIF (LmjMIF1 and LmjMIF2) (57). Both LmjMIF and LmjMIF2 crystallize in a trimeric form similar to that of mammalian MIF, but only LmjMIF1 is enzymatically active. Comparison of the X-ray structure revealed subtle structural differences between the two isoforms, mainly in the N-terminal region comprising residues 30–37. Interestingly, C-terminal mutations that abolish the

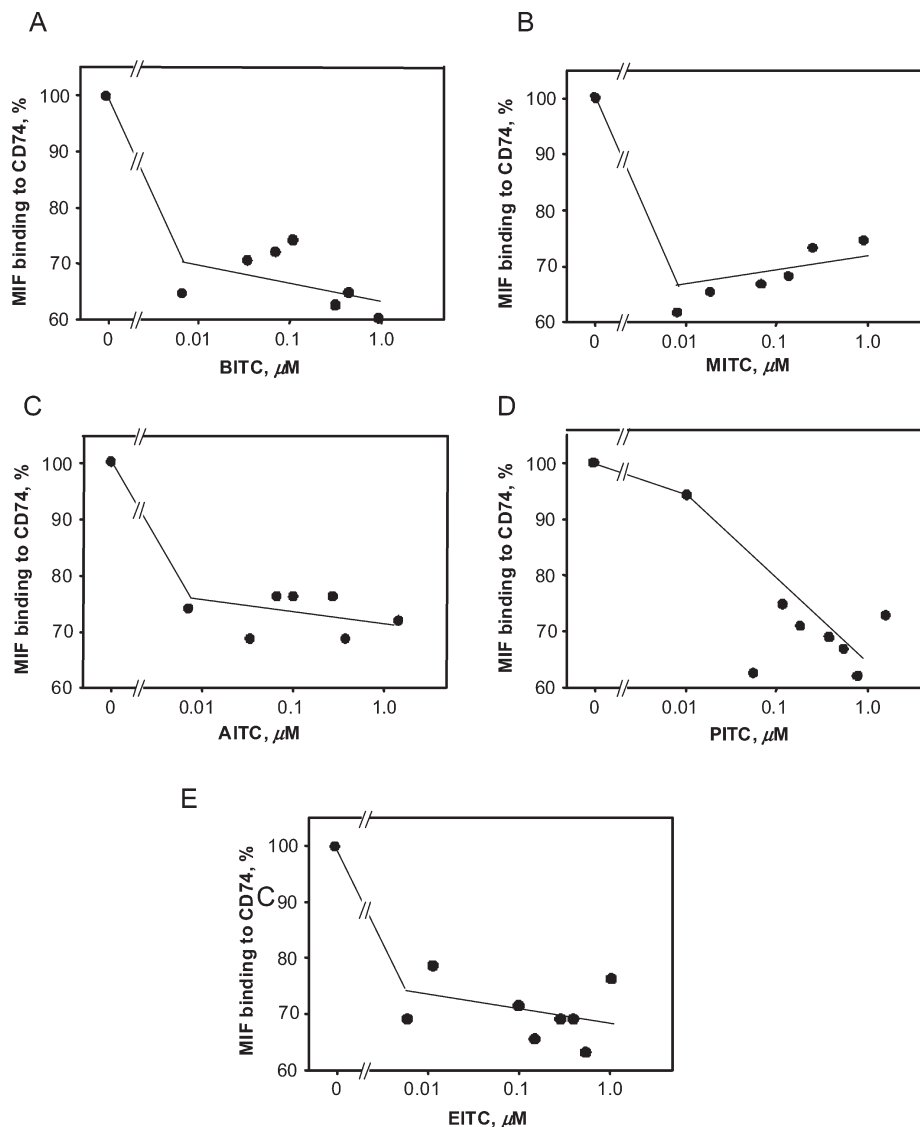


FIGURE 8: ITC-based inhibitors of MIF tautomerase activity interfere with binding of MIF to its receptor, CD74. Dose-dependent inhibition by isothiocyanate derivatives (A) BITC, (B) AITC, (C) EITC, (D) MITC, and (E) 2PITC. Test compounds were preincubated with $2 \text{ ng}/\mu\text{L}$ of $0.2 \mu\text{M}$ biotinylated MIF equal to $0.16 \mu\text{M}$ for 2 h at room temperature in the dark prior to addition to 96-well plates precoated with recombinant human sCD74. Data points are the means of duplicate determinations of bound MIF.

catalytic activity of MIF favor the formation of a trimeric conformation that is distinct from those populated by the wild-type protein (43). Studies by Bucala and colleagues demonstrated that covalent modification or mutation of Pro1 induces structural changes at the N-terminus that alter its recognition by monoclonal antibodies and result in a significant reduction in MIF cell surface binding activity (31). These findings demonstrate that MIF is capable of adopting different trimeric states that seem to differ primarily at the levels of tertiary structure, catalytic activity, and receptor binding activity.

Previously, we showed that C-terminal deletion mutants (ΔC5 huMIF_{1–109} and ΔC10 huMIF_{1–104}) and a mutant in which the secondary structure of the C-terminal region comprising residues 104–114 has been disrupted by the insertion of proline at position 107 (P107 huMIF) are catalytically inactive and elute as a single trimeric peak which elutes earlier than the unmodified protein. Unlike the BITC-modified MIF, all the C-terminal deletion mutants (ΔC5 huMIF_{1–109} and ΔC10 huMIF_{1–104}) exhibited virtually identical binding to CD74. These findings suggest that C-terminal residues 105–114 may not be directly

involved in the binding to CD74 and further support a direct relationship between the N-terminal region in modulating the structural properties and protein–protein interactions mediating the biological activity of MIF. Together, our results combined with the lack of data supporting the existence of a stable monomeric MIF highlight the role of tertiary structure rather than quaternary structure in modulating the biochemical and biological activities of MIF. At this stage, we cannot rule out a biological role for monomeric MIF *in vivo* and the possibility that changes in the oligomerization state may also constitute a molecular switch for modulating different functions of MIF.

Our observation that BITC and its analogues modify only enzymatically active conformations of MIF and induce further conformational changes that alter MIF receptor binding properties highlights the potential of new strategies for monitoring and/or modulating MIF's activity in health and disease. Conformational changes that alter the active site or pK_a of the catalytic proline residue appear to be sufficient for abolishing MIF reactivity toward BITC and other ITCs. These results suggest that altering the catalytic site via selective modification of the

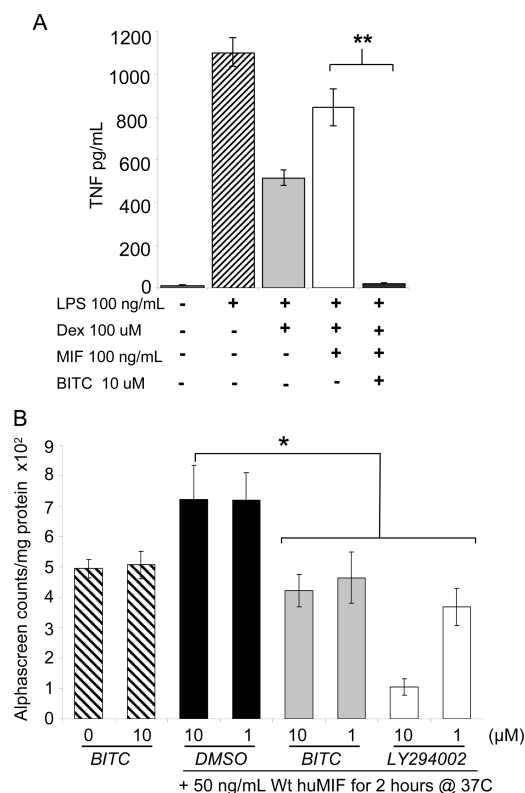


FIGURE 9: Effect of BITC on MIF-mediated glucocorticoid over-riding activity (A) and Akt phosphorylation (B). (A) Human macrophages were preincubated for 1 h with (+) or without (–) dexamethasone (Dex 100 nM), MIF (100 ng/mL), and BITC (10 μ M) before stimulation with LPS (100 ng/mL). Cell-free supernatants were collected after 4 h to quantify the concentrations of TNF. Data are means \pm the standard deviation of triplicate samples from one experiment and are representative of four independent experiments. Statistical analyses were performed using one-way ANOVA followed by Scheffé's test. $**p < 0.001$. (B) BITC inhibits MIF-induced activation of Akt phosphorylation. HeLa cells were incubated for 2 h with 50 ng/mL MIF in the presence or absence of 1–10 μ M BITC and LY294002 (a selective inhibitor of the PI3K/Akt pathway). Akt phosphorylation was quantified using the Alpha screen SureFire phosphokinase kit as described in Materials and Methods. Data are expressed as Alpha screen counts per milligram of total protein (mean \pm standard deviation of triplicate samples from one experiment and representative of two independent experiments). Statistical analyses were performed using one-way ANOVA followed by Scheffé's test. $*p < 0.05$.

N-terminal proline residue may present new opportunities for modulating MIF biological activities in vivo. More recently, Merk et al. also demonstrated that covalent modification of Pro1 by 4-iodo-6-phenylpyrimidine (4-IPP) blocks MIF secretion by influencing its interaction with p115, a Golgi complex-associated protein recently identified as a key partner of MIF and modulator of its secretion (58). A change in the native conformation of MIF was proposed as a mechanism underlying the effect of 4-IPP on MIF–p115 interaction, although the nature of this change was not investigated. These findings are consistent with our results and demonstrate the possibility of regulating MIF's catalytic activity and suggest different roles for the catalytic domain and tautomerase activity.

CONCLUSION

We have shown that BITC and other ITCs are effective inhibitors of MIF tautomerase activity. Although this inhibitory

effect of ITCs is mediated by their covalent modification of the catalytic N-terminal proline residue, their effect on MIF's receptor binding and proinflammatory activity is in part mediated by changes in the tertiary structure of MIF as a result of modification of the N-terminus and/or catalytic site. The design of novel active site-directed irreversible inhibitors based on structural complementarity with the catalytic active site and incorporation of reactive groups should yield novel mechanism-based inhibitors of MIF biological activities. Furthermore, the specificity and selectivity of BITC reactivity with Pro1, as a consequence of its low pK_a , also open new opportunities for specific labeling of MIF and monitoring of its activity in vivo. For example, MIF reactivity with ITCs could be used as an indirect method to quantify the ratio of active to inactive MIF trimers in biological samples, which might provide a better correlation with disease progression and severity than total MIF levels.

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SUPPORTING INFORMATION AVAILABLE

BITC modification of MIF (Figure S1), BITC modification specificity toward lysozyme or α -synuclein and ubiquitin (Figure S2), and ITC toxicity studies (Figure S3). This material is available free of charge via the Internet at <http://pubs.acs.org>.

REFERENCES

- Calandra, T., and Roger, T. (2003) Macrophage migration inhibitory factor: A regulator of innate immunity. *Nat. Rev. Immunol.* **3**, 791–800.
- Calandra, T., Bernhagen, J., Metz, C. N., Spiegel, L. A., Bacher, M., Donnelly, T., Cerami, A., and Bucala, R. (1995) MIF as a glucocorticoid-induced modulator of cytokine production. *Nature* **377**, 68–71.
- Mitchell, R. A., Metz, C. N., Peng, T., and Bucala, R. (1999) Sustained mitogen-activated protein kinase (MAPK) and cytoplasmic phospholipase A2 activation by macrophage migration inhibitory factor (MIF). Regulatory role in cell proliferation and glucocorticoid action. *J. Biol. Chem.* **274**, 18100–18106.
- Hudson, J. D., Shoaibi, M. A., Maestro, R., Carnero, A., Hannon, G. J., and Beach, D. H. (1999) A proinflammatory cytokine inhibits p53 tumor suppressor activity. *J. Exp. Med.* **190**, 1375–1382.
- Fingerle-Rowson, G., Petrenko, O., Metz, C. N., Forsthuber, T. G., Mitchell, R., Huss, R., Moll, U., Muller, W., and Bucala, R. (2003) The p53-dependent effects of macrophage migration inhibitory factor revealed by gene targeting. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 9354–9359.
- Lue, H., Kapurniotu, A., Fingerle-Rowson, G., Roger, T., Leng, L., Thiele, M., Calandra, T., Bucala, R., and Bernhagen, J. (2006) Rapid and transient activation of the ERK MAPK signalling pathway by macrophage migration inhibitory factor (MIF) and dependence on JAB1/CAN5 and Src kinase activity. *Cell. Signalling* **18**, 688–703.
- Morand, E. F., Leech, M., Weedon, H., Metz, C., Bucala, R., and Smith, M. D. (2002) Macrophage migration inhibitory factor in rheumatoid arthritis: Clinical correlations. *Rheumatology (Oxford, U.K.)* **41**, 558–562.
- Radstake, T. R., Nabbe, K. C., Wenink, M. H., Roelofs, M. F., Oosterlaar, A., van Lieshout, A. W., Barrera, P., van Lent, P. L., and van den Berg, W. B. (2005) Dendritic cells from patients with rheumatoid arthritis lack the interleukin 13 mediated increase of Fc gamma RII expression, which has clear functional consequences. *Ann. Rheum. Dis.* **64**, 1737–1743.
- Lan, H. Y., Yang, N., Nikolic-Paterson, D. J., Yu, X. Q., Mu, W., Isbel, N. M., Metz, C. N., Bucala, R., and Atkins, R. C. (2000)

- Expression of macrophage migration inhibitory factor in human glomerulonephritis. *Kidney Int.* 57, 499–509.
10. Brown, F. G., Nikolic-Paterson, D. J., Hill, P. A., Isbel, N. M., Dowling, J., Metz, C. M., and Atkins, R. C. (2002) Urine macrophage migration inhibitory factor reflects the severity of renal injury in human glomerulonephritis. *J. Am. Soc. Nephrol.* 13 (Suppl. 1), S7–S13.
 11. Yabunaka, N., Nishihira, J., Mizue, Y., Tsuji, M., Kumagai, M., Ohtsuka, Y., Imamura, M., and Asaka, M. (2000) Elevated serum content of macrophage migration inhibitory factor in patients with type 2 diabetes. *Diabetes Care* 23, 256–258.
 12. Verschuren, L., Kooistra, T., Bernhagen, J., Voshol, P. J., Ouwens, D. M., van Erk, M., de Vries-van der Weij, J., Leng, L., van Bockel, J. H., van Dijk, K. W., Fingerle-Rowson, G., Bucala, R., and Kleemann, R. (2009) MIF Deficiency Reduces Chronic Inflammation in White Adipose Tissue and Impairs the Development of Insulin Resistance, Glucose Intolerance, and Associated Atherosclerotic Disease. *Circ. Res.* 105, 99–107.
 13. de Jong, Y. P., Abadia-Molina, A. C., Satoskar, A. R., Clarke, K., Rietdijk, S. T., Faubion, W. A., Mizoguchi, E., Metz, C. N., Alsahli, M., ten Hove, T., Keates, A. C., Lubetsky, J. B., Farrell, R. J., Michetti, P., van Deventer, S. J., Lolis, E., David, J. R., Bhan, A. K., and Terhorst, C. (2001) Development of chronic colitis is dependent on the cytokine MIF. *Nat. Immunol.* 2, 1061–1066.
 14. Bernhagen, J., Calandra, T., Mitchell, R. A., Martin, S. B., Tracey, K. J., Voelker, W., Manogue, K. R., Cerami, A., and Bucala, R. (1993) MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. *Nature* 365, 756–759.
 15. Roger, T., Glauser, M. P., and Calandra, T. (2001) Macrophage migration inhibitory factor (MIF) modulates innate immune responses induced by endotoxin and Gram-negative bacteria. *J. Endotoxin Res.* 7, 456–460.
 16. Beishuizen, A., Thijs, L. G., Haanen, C., and Vermes, I. (2001) Macrophage migration inhibitory factor and hypothalamo-pituitary-adrenal function during critical illness. *J. Clin. Endocrinol. Metab.* 86, 2811–2816.
 17. Emonts, M., Sweep, F. C., Grebentchikov, N., Geurts-Moespot, A., Knaap, M., Chanson, A. L., Erard, V., Renner, P., Hermans, P. W., Hazelzet, J. A., and Calandra, T. (2007) Association between high levels of blood macrophage migration inhibitory factor, inappropriate adrenergic response, and early death in patients with severe sepsis. *Clin. Infect. Dis.* 44, 1321–1328.
 18. Arjona, A., Foellmer, H. G., Town, T., Leng, L., McDonald, C., Wang, T., Wong, S. J., Montgomery, R. R., Fikrig, E., and Bucala, R. (2007) Abrogation of macrophage migration inhibitory factor decreases West Nile virus lethality by limiting viral neuroinvasion. *J. Clin. Invest.* 117, 3059–3066.
 19. Donnelly, S. C., Haslett, C., Reid, P. T., Grant, I. S., Wallace, W. A., Metz, C. N., Bruce, L. J., and Bucala, R. (1997) Regulatory role for macrophage migration inhibitory factor in acute respiratory distress syndrome. *Nat. Med.* 3, 320–323.
 20. Mizue, Y., Ghani, S., Leng, L., McDonald, C., Kong, P., Baugh, J., Lane, S. J., Craft, J., Nishihira, J., Donnelly, S. C., Zhu, Z., and Bucala, R. (2005) Role for macrophage migration inhibitory factor in asthma. *Proc. Natl. Acad. Sci. U.S.A.* 102, 14410–14415.
 21. Mitchell, R. A. (2004) Mechanisms and effectors of MIF-dependent promotion of tumorigenesis. *Cell. Signalling* 16, 13–19.
 22. Meyer-Siegler, K. L., Vera, P. L., Iczkowski, K. A., Bifulco, C., Lee, A., Gregersen, P. K., Leng, L., and Bucala, R. (2007) Macrophage migration inhibitory factor (MIF) gene polymorphisms are associated with increased prostate cancer incidence. *Genes Immun.* 8, 646–652.
 23. Bucala, R., and Donnelly, S. C. (2007) Macrophage migration inhibitory factor: A probable link between inflammation and cancer. *Immunity* 26, 281–285.
 24. Rosengren, E., Bucala, R., Aman, P., Jacobsson, L., Odh, G., Metz, C. N., and Rorsman, H. (1996) The immunoregulatory mediator macrophage migration inhibitory factor (MIF) catalyzes a tautomerization reaction. *Mol. Med.* 2, 143–149.
 25. Lolis, E., and Bucala, R. (1996) Crystal structure of macrophage migration inhibitory factor (MIF), a glucocorticoid-induced regulator of cytokine production, reveals a unique architecture. *Proc. Assoc. Am. Physicians* 108, 415–419.
 26. Bendrat, K., Al-Abed, Y., Callaway, D. J., Peng, T., Calandra, T., Metz, C. N., and Bucala, R. (1997) Biochemical and mutational investigations of the enzymatic activity of macrophage migration inhibitory factor. *Biochemistry* 36, 15356–15362.
 27. Stamps, S. L., Fitzgerald, M. C., and Whitman, C. P. (1998) Characterization of the role of the amino-terminal proline in the enzymatic activity catalyzed by macrophage migration inhibitory factor. *Biochemistry* 37, 10195–10202.
 28. Al-Abed, Y., Dabideen, D., Aljabari, B., Valster, A., Messmer, D., Ochani, M., Tanovic, M., Ochani, K., Bacher, M., Nicoletti, F., Metz, C., Pavlov, V. A., Miller, E. J., and Tracey, K. J. (2005) ISO-1 binding to the tautomerase active site of MIF inhibits its pro-inflammatory activity and increases survival in severe sepsis. *J. Biol. Chem.* 280, 36541–36544.
 29. Crichlow, G. V., Cheng, K. F., Dabideen, D., Ochani, M., Aljabari, B., Pavlov, V. A., Miller, E. J., Lolis, E., and Al-Abed, Y. (2007) Alternative chemical modifications reverse the binding orientation of a pharmacophore scaffold in the active site of macrophage migration inhibitory factor. *J. Biol. Chem.* 282, 23089–23095.
 30. Morand, E. F. (2005) New therapeutic target in inflammatory disease: Macrophage migration inhibitory factor. *Intern. Med. J.* 35, 419–426.
 31. Senter, P. D., Al-Abed, Y., Metz, C. N., Benigni, F., Mitchell, R. A., Chesney, J., Han, J., Gartner, C. G., Nelson, S. D., Todaro, G. J., and Bucala, R. (2002) Inhibition of macrophage migration inhibitory factor (MIF) tautomerase and biological activities by acetaminophen metabolites. *Proc. Natl. Acad. Sci. U.S.A.* 99, 144–149.
 32. Lubetsky, J. B., Dios, A., Han, J., Aljabari, B., Ruzsicska, B., Mitchell, R., Lolis, E., and Al-Abed, Y. (2002) The tautomerase active site of macrophage migration inhibitory factor is a potential target for discovery of novel anti-inflammatory agents. *J. Biol. Chem.* 277, 24976–24982.
 33. Dios, A., Mitchell, R. A., Aljabari, B., Lubetsky, J., O'Connor, K., Liao, H., Senter, P. D., Manogue, K. R., Lolis, E., Metz, C., Bucala, R., Callaway, D. J., and Al-Abed, Y. (2002) Inhibition of MIF bioactivity by rational design of pharmacological inhibitors of MIF tautomerase activity. *J. Med. Chem.* 45, 2410–2416.
 34. Dabideen, D. R., Cheng, K. F., Aljabari, B., Miller, E. J., Pavlov, V. A., and Al-Abed, Y. (2007) Phenolic hydrazones are potent inhibitors of macrophage migration inhibitory factor proinflammatory activity and survival improving agents in sepsis. *J. Med. Chem.* 50, 1993–1997.
 35. Bernhagen, J., Mitchell, R. A., Calandra, T., Voelker, W., Cerami, A., and Bucala, R. (1994) Purification, bioactivity, and secondary structure analysis of mouse and human macrophage migration inhibitory factor (MIF). *Biochemistry* 33, 14144–14155.
 36. Philo, J. S., Yang, T. H., and LaBarre, M. (2004) Re-examining the oligomerization state of macrophage migration inhibitory factor (MIF) in solution. *Biophys. Chem.* 108, 77–87.
 37. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) NMRPipe: A multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* 6, 277–293.
 38. Muhlhahn, P., Bernhagen, J., Czisch, M., Georgescu, J., Renner, C., Ross, A., Bucala, R., and Holak, T. A. (1996) NMR characterization of structure, backbone dynamics, and glutathione binding of the human macrophage migration inhibitory factor (MIF). *Protein Sci.* 5, 2095–2103.
 39. Grzesiek, S., Stahl, S. J., Wingfield, P. T., and Bax, A. (1996) The CD4 determinant for downregulation by HIV-1 Nef directly binds to Nef. Mapping of the Nef binding surface by NMR. *Biochemistry* 35, 10256–10261.
 40. Leng, L., Metz, C. N., Fang, Y., Xu, J., Donnelly, S., Baugh, J., Delohery, T., Chen, Y., Mitchell, R. A., and Bucala, R. (2003) MIF signal transduction initiated by binding to CD74. *J. Exp. Med.* 197, 1467–1476.
 41. Meyer, D. J., Crease, D. J., and Ketterer, B. (1995) Forward and reverse catalysis and product sequestration by human glutathione S-transferases in the reaction of GSH with dietary aralkyl isothiocyanates. *Biochem. J.* 306 (Part 2), 565–569.
 42. Ralat, L. A., and Colman, R. F. (2004) Glutathione S-transferase Pi has at least three distinguishable xenobiotic substrate sites close to its glutathione-binding site. *J. Biol. Chem.* 279, 50204–50213.
 43. El-Turk, F., Cascella, M., Ouertatani-Sakouhi, H., Narayanan, R. L., Leng, L., Bucala, R., Zweckstetter, M., Rothlisberger, U., and Lashuel, H. A. (2008) The Conformational Flexibility of the Carboxy Terminal Residues 105–114 Is a Key Modulator of the Catalytic Activity and Stability of Macrophage Migration Inhibitory Factor. *Biochemistry* 47, 10740–10756.
 44. Swope, M. D., Sun, H. W., Klockow, B., Blake, P., and Lolis, E. (1998) Macrophage migration inhibitory factor interactions with glutathione and S-hexylglutathione. *J. Biol. Chem.* 273, 14877–14884.
 45. Shi, X., Leng, L., Wang, T., Wang, W., Du, X., Li, J., McDonald, C., Chen, Z., Murphy, J. W., Lolis, E., Noble, P., Knudson, W., and Bucala, R. (2006) CD44 is the signaling component of the macrophage migration inhibitory factor-CD74 receptor complex. *Immunity* 25, 595–606.

46. Cournia, Z., Leng, L., Gandavadi, S., Du, X., Bucala, R., and Jorgensen, W. L. (2009) Discovery of human macrophage migration inhibitory factor (MIF)-CD74 antagonists via virtual screening. *J. Med. Chem.* *52*, 416–424.
47. Kamir, D., Zierow, S., Leng, L., Cho, Y., Diaz, Y., Griffith, J., McDonald, C., Merk, M., Mitchell, R. A., Trent, J., Chen, Y., Kwong, Y. K., Xiong, H., Vermeire, J., Cappello, M., McMahon-Pratt, D., Walker, J., Bernhagen, J., Lolis, E., and Bucala, R. (2008) A *Leishmania* ortholog of macrophage migration inhibitory factor modulates host macrophage responses. *J. Immunol.* *180*, 8250–8261.
48. Lue, H., Thiele, M., Franz, J., Dahl, E., Speckgens, S., Leng, L., Fingerle-Rowson, G., Bucala, R., Luscher, B., and Bernhagen, J. (2007) Macrophage migration inhibitory factor (MIF) promotes cell survival by activation of the Akt pathway and role for CSN5/JAB1 in the control of autocrine MIF activity. *Oncogene* *26*, 5046–5059.
49. Golubkov, P. A., Johnson, W. H., Jr., Czerwinski, R. M., Person, M. D., Wang, S. C., Whitman, C. P., and Hackert, M. L. (2006) Inactivation of the phenylpyruvate tautomerase activity of macrophage migration inhibitory factor by 2-oxo-4-phenyl-3-butynoate. *Bioorg. Chem.* *34*, 183–199.
50. Winner, M., Meier, J., Zierow, S., Rendon, B. E., Crichlow, G. V., Riggs, R., Bucala, R., Leng, L., Smith, N., Lolis, E., Trent, J. O., and Mitchell, R. A. (2008) A novel, macrophage migration inhibitory factor suicide substrate inhibits motility and growth of lung cancer cells. *Cancer Res.* *68*, 7253–7257.
51. Higdon, J. V., Delage, B., Williams, D. E., and Dashwood, R. H. (2007) Cruciferous vegetables and human cancer risk: Epidemiologic evidence and mechanistic basis. *Pharmacol. Res.* *55*, 224–236.
52. Miyoshi, N., Takabayashi, S., Osawa, T., and Nakamura, Y. (2004) Benzyl isothiocyanate inhibits excessive superoxide generation in inflammatory leukocytes: Implication for prevention against inflammation-related carcinogenesis. *Carcinogenesis* *25*, 567–575.
53. Miyoshi, N., Uchida, K., Osawa, T., and Nakamura, Y. (2004) A link between benzyl isothiocyanate-induced cell cycle arrest and apoptosis: Involvement of mitogen-activated protein kinases in the Bcl-2 phosphorylation. *Cancer Res.* *64*, 2134–2142.
54. Mi, L., Gan, N., Cheema, A., Dakshanamurthy, S., Yang, D. C., and Chung, F. L. (2009) Cancer preventive isothiocyanates induce selective degradation of cellular α - and β -tubulins by proteasomes. *J. Biol. Chem.* *284*, 17039–17051.
55. Tsou, M. F., Peng, C. T., Shih, M. C., Yang, J. S., Lu, C. C., Chiang, J. H., Wu, C. L., Lin, J. P., Lo, C., Fan, M. J., and Chung, J. G. (2009) Benzyl isothiocyanate inhibits murine WEHI-3 leukemia cells in vitro and promotes phagocytosis in BALB/c mice in vivo. *Leuk. Res.* *33*, 1505–1511.
56. Fingerle-Rowson, G., Kaleswarapu, D. R., Schlander, C., Kabgani, N., Brocks, T., Reinart, N., Busch, R., Schutz, A., Lue, H., Du, X., Liu, A., Xiong, H., Chen, Y., Nemajerova, A., Hallek, M., Bernhagen, J., Leng, L., and Bucala, R. (2009) A tautomerase-null macrophage migration-inhibitory factor (MIF) gene knock-in mouse model reveals that protein interactions and not enzymatic activity mediate MIF-dependent growth regulation. *Mol. Cell. Biol.* *29*, 1922–1932.
57. Richardson, J. M., Morrison, L. S., Bland, N. D., Bruce, S., Coombs, G. H., Mottram, J. C., and Walkinshaw, M. D. (2009) Structures of *Leishmania major* orthologues of macrophage migration inhibitory factor. *Biochem. Biophys. Res. Commun.* *380*, 442–448.
58. Merk, M., Baugh, J., Zierow, S., Leng, L., Pal, U., Lee, S. J., Ebert, A. D., Mizue, Y., Trent, J. O., Mitchell, R., Nickel, W., Kavathas, P. B., Bernhagen, J., and Bucala, R. (2009) The Golgi-associated protein p115 mediates the secretion of macrophage migration inhibitory factor. *J. Immunol.* *182*, 6896–6906.
59. Goddard, T. D., and Kneller, D. G. (2004) SPARKY 3, University of California, San Francisco.